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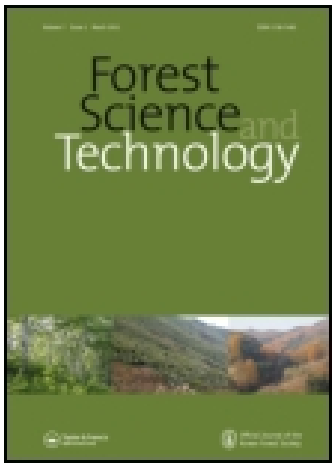

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Potassium Chloride Elicits Enhancement of Bilobalide and Ginkgolides Production by *Ginkgo biloba* Cell Cultures

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This study investigated the ability of potassium chloride (KCl) to elicit the production of bilobalide (BB), ginkgolide A (GA) and ginkgolide B (GB) by *Ginkgo biloba* cell suspension cultures. The salt stress by KCl treatments increased production of BB, GA and GB in both suspended cells and cultured medium. Especially, treatment of KCl 800 mM of highest concentration was stimulated emission into cultured medium BB, GA and GB compounds accumulated in cells. Although KCl 800 mM severely inhibited cells growth, the maximum content of GA and GB in cells was obtained in the treatment of KCl 800 mM, which was 1.9 and 4.0 times higher than the control. These results thus suggest that salt stress can afford enhanced production of secondary metabolites by plant cell cultures.

Key words **:** Ginkgo biloba, *potassium chloride (KCl), cell culture, ginkgolide, bilobalide, abiotic elicitor*

INTRODUCTION

Ginkgo biloba is one of the oldest plants with a long history of use in traditional Chinese medicine. It is a rich source of diverse phytochemicals like flavonoids, terpenoids, and other compounds (Huh *et al.*, 1992; Kang *et al.*, 1990). Of these ingredients, ginkgolides (GA and GB) and bilobalide (BB) (Figure 1) are beneficial to human body (Laurain *et al.*, 1997). BB is a sesquiterpene and GA and GB are diterpenes (Carrier *et al.*, 1998). In particular, GB has strong antagonist effects against platelet activating factors (PAF) related to the development of a number of cardiovascular, renal, respiratory and central nervous system disorders (Smith *et al.*, 1996).

In recent years, ginkgo leaf extracts have been widely sold as phytomedicines in Europe and as dietary supplements worldwide. However, there remain several difficulties in meeting the demand of ginkgo extracts. Cultivation of the trees to harvest is lengthy, and the content of BB and GA/GB are highly variable depending both on season and on the sex of the trees. Additionally, leaf collection is labor intensive, and the other alternative such as chemical synthesis of gingkolides is difficult (Kim *et al.*, 1996). Generally it has been observed that GB contents in ginkgo extracts from cultivated trees are very low compared to other compounds B and GA. Therefore, to provide a sufficient supply of these compounds, strategies to enhance their production by *in vitro* culture methods are needed.

Over the past years, economically important plants have been brought into cell cultures, but in most cases the productivity was too low to allow for an economically feasible process (Misawa and Nakanishio, 1998). However, the concentrations of the sec-

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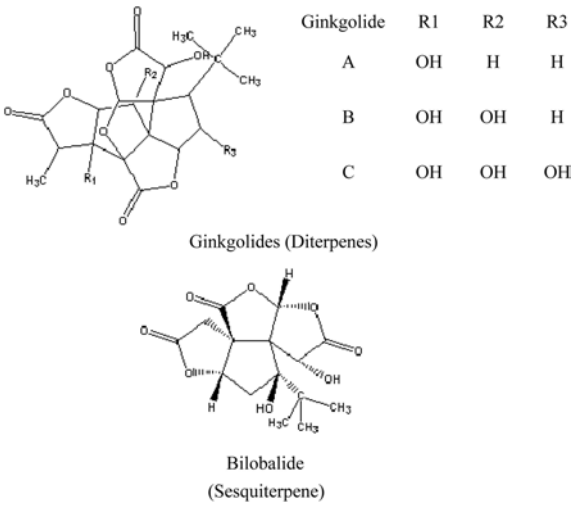

Figure 1. Structure of ginkgolides and bilobalide.

ondary metabolites from mother plants did not agree with the cultured cells. Numerous researches have reported that the concentrations of the secondary metabolites in the cultured cells were lower than those in the mother plants. Although the productivity in most cell culture cases was too low to allow an economically feasible process (Misawa and Nakanishio, 1998), selection of cell line accumulating high content and introduction of various biotechnological strategies in the selected cell line are expected to enhance the capability for high phytochemical production.

Elicitation is an attractive strategy for increasing the metabolite productivity *in vitro* culture system. The elicitation of plant cells and tissue can lead to increased yields and can shorten the metabolite generation time. Elicitors of nonbiological (abiotic) origin include heavy metals, salts, RNase and ultraviolet radiation (Discosmo and Misawa, 1985). Secondary metabolites accumulate in plant cell cultures in response to abiotic elicitor encounter. Inorganic salts are readily available, economical and easy to use, and are also chemically defined. It has been proposed that the treatment of plant cell cultures with inorganic salts and heavy metals may be an ideal method of elicitation for commercial production of secondary metabolites and phytoalexins. In addition to abiotic elicitation, the stress in plant can be an effective approach to increase the metabolites. Plant defense mechanism and metabolite production are generally shown to be correlated because of secondary metabolism (Sahai and Shuler, 1984). Here we report the influence of Potassium chloride (KCl) salt stress on the enhanced production of ginkgolides by *G. biloba* cell suspension cultures.

MATERIALS AND METHODS

G. biloba callus induction and cell suspension culture

The callus of *G. biloba* was induced from various tree parts such as embryo, leaf, stem and root. The surface of seed was sterilized with 70% (v/v) ethanol for 1 min, 3% (v/v) NaClO for 15 min and then rinsed with sterile distilled water more than 7 times. The embryo was expelled from sterilized seed with knife and pincette. The embryo surface was wounded with knife and placed on sterile MS solid medium (pH 5.8) supplemented with 3% (w/v) sucrose, 0.3% (w/v) gelrite and 3.5 mg/L NAA to induce callus. The callus was also induced from leaf, stem and root of *in vitro* plant. Each explants was cut into segments of 2–3 cm length, and placed on same medium as callus induced from embryo and incubated under dark condition at 25°C, and sub-cultured at 4 week intervals. To proliferate, induced callus was transferred into MS liquid medium supplemented with 3 % (w/v) sucrose and 3.5 mg/L NAA. The cell suspension cultures were maintained at 100 rpm and 25°C under dark condition.

Elicitor treatment of *G. biloba* cell cultures

Inorganic salt, KCl was employed as an abiotic elicitor for cell cultures. Aqueous KCl solutions of various concentrations (50, 200 and 800 mM) were prepared in distilled water and sterilized by autoclaving at 121°C for 15 min. The salt stress was applied to 14-day-old cell suspension cultures by exposing for 12, 24, 48 and 72 h.

Measurement of *G. biloba* cell growth

The effect of the salt treatment was assessed by measuring cell growth. *G. biloba* suspension cell growth was measured by determining fresh and dry weights. To gain the fresh weight (F.W.) the cells were separated from the medium by filtration, washed repeatedly with distilled water and weighed. The dry weight (D.W.) of the cells was recorded after drying at 50°C for 24 h.

Extraction and quantification of phytochemicals

The effect of salt treatment on the production of BB, GA and GB in *G. biloba* cell cultures was determined by extraction and quantification of Kang *et al* (2006). Dried cells (0.1 g) were ground with pestle and mortar and extracted with ethyl acetate. Samples of the cells (0.1g) were extracting

with 10 mL ethyl acetate for 2 h in ultra-sonicator. The extracts were then centrifuged at 6,000 rpm for 10 min, and the supernatant solution was concentrated using a rotary vacuum evaporator. BB, GA and GB in the cultured medium were extracted with ethyl acetate in equal volumes by intermittent vortexing over 2 days. The combined ethyl acetate phase was evaporated using the rotary vacuum evaporator. The residue that was obtained was dissolved in 200 μL MeOH (HPLC grade), filtered through a pre-filter (ψ0.2 μm Supelco) and analyzed and quantified by HPLC (Kang *et al.*, 2006).

Statistical analysis

The experiments were repeated for a minimum of three times. Each numerical value represents the mean and standard deviation (SD) by analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Effect of KCl salt stress on *G. biloba* cell growth

KCl salt treatment was detrimental to *G. biloba* cell growth (Figure 2). The cells at 24 h after elicitation with KCl 800 mM were severely inhibited cell growth and the cells color turned into grayish at 48 h. About 22% of decrease in *G. biloba* cell growth was observed after KCl 800 mM treatment in 48 h compared to control. In similar experiments the decrease in cell growth was approximately 9 and 15% when KCl 50 and 200 mM were used as an elicitor after at the end of 48 h. However, slight decrease of cell growth was observed at 24 h after treatment of *G. biloba* cells with KCl 50 mM. In appearance the cells were not indicated change of cell surface color by the concentration of 50 mM salt for 24 h after elicitation. KCl and sodium chloride (NaCl) have been studied for their effects on growth and secondary metabolite production as abiotic elicitor in plants (Jeong and Park, 2006; Kim and Roh, 1998). During salt stress, in addition to the osmotic stress and ion toxicity the plants also experience an oxidative stress and all of these contribute to the observed deleterious effects (Gosset *et al.*, 1996). The adverse effect of NaCl has been attributed to changes in osmotic potential resulting from reducing water content. The effects may also be due to specific toxic effects caused by the accumulation of sodium and chloride ions as observed in the case of *Suaeda maritime*, *Suaeda monoica* and other plants (Ali, 2000).

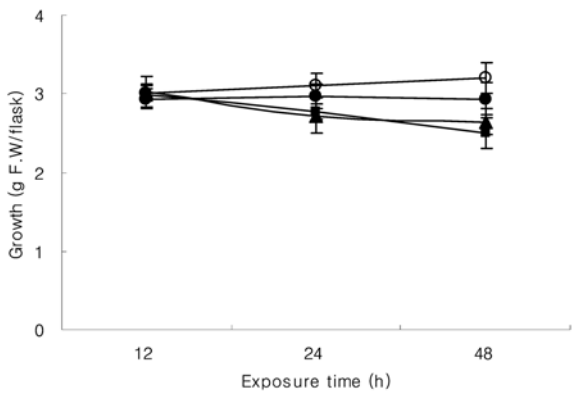

Figure 2. Effect of KCl treatment on growth by cell suspension culture of *G. biloba*. (●: 50 mM KCl, ▲: 200 mM KCl, ■: 800 mM KCl, ○: control).

KCl elicits accumulation of bilobalide and ginkgolides in *G. biloba* cells

The production pattern of BB, GA and GB by cell cultures treated with KCl as an abiotic elicitor was compared. KCl was introduced into the cultured medium after 14 day (exposure time 0 h) and effects were observed at 16 day (exposure time 48 h).

The production of BB, GA and GB was compared after 48 h exposure of various KCl solutions (Figure 3). After treatment with KCl the BB content in cells decreased (Figure 3A). The treatment of KCl 50 mM influenced decrease of 6.9 times compared to the control at 48 h of exposure time. Similarly BB production also decreased after treatment with KCl 200 mM and 800 mM. The fact that the BB production in cells was decreased following treatments with KCl 50, 200 and 800 mM is consistent with a stress response of plant cells to KCl. Although these attempts were expected to release BB out of cells by KCl treatment, but on the contrary, GA and GB production were enhanced after salt stress (Figure 3 B and C). After treatment of cells with KCl 800 mM, the GA content in the cells increased and reached a maximum level after 48 h of exposure yielding a maximum of 15.3 mg/g D.W. which is approximately 2 times higher compared with control. However, other concentrations of KCl did not mediate an increase the accumulation of GA.

Among the treatments, KCl 800 mM elevated the GB production at 48 h of exposure time. The treatment with KCl 200 mM was effective for the enhanced production of GB at 48 h. However KCl 50 mM did not influence enhancements of BB, GA and GB production. Although KCl 800 mM severely inhibited cells growth, it was effective in production

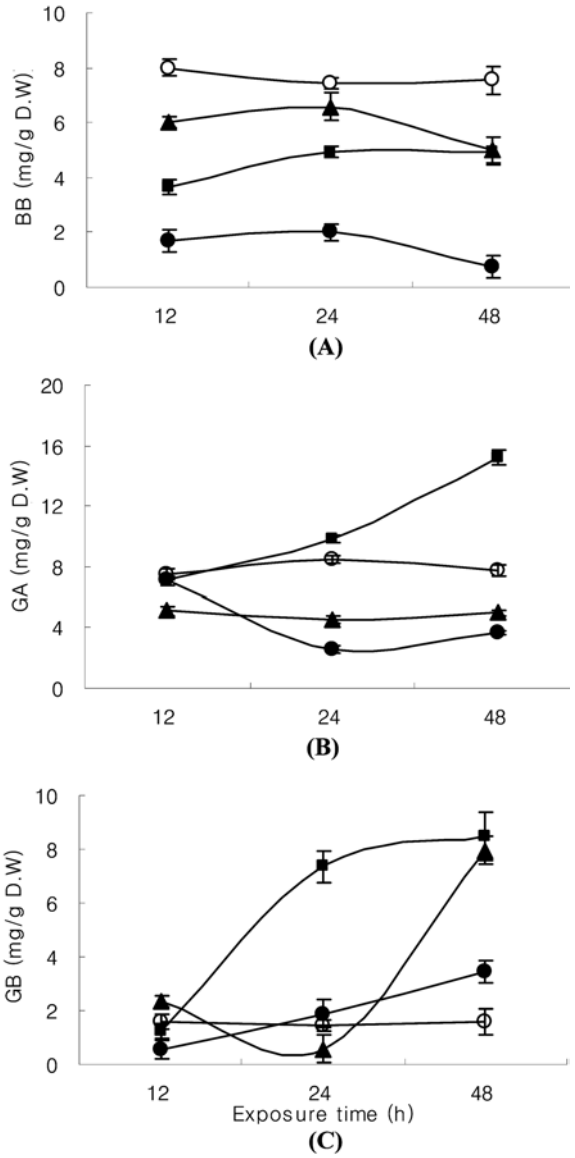

Figure 3. Effects of KCl on the production of bilobalide (A), ginkgolide A (B) and ginkgolide B (C) production in suspended cells of *G. bilboa*. (●: 50 mM KCl, ▲: 200 mM KCl, ■: 800 mM KCl, ○: control).

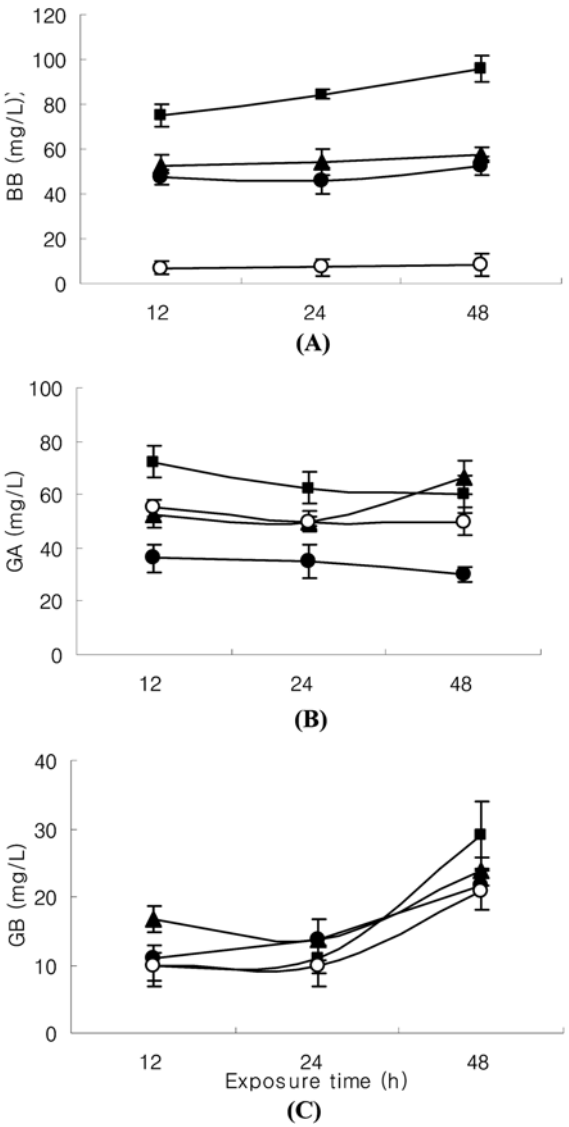

Figure 4. Accumulations of bilobalide (A), ginkgolide A (B) and ginkgolide B (C) in cell cultured medium on treatment with KCl. (●: 50 mM KCl, ▲: 200 mM KCl, ■: 800 mM KCl, ○: control)

of more GA and GB in cells corresponding respectively up to 1.9 and 4.0 times than that of control.

KCl elicits an accumulation of bilobalide and ginkgolides in cultured medium

Treatment of KCl not only increased the production of GA and GB in cells, but also markedly released them into the cultured medium (Fig. 4). The treatment of KCl 800 mM stimulated a release of BB up to 9.8 times than control after 48 h of exposure. KCl 800 mM also stimulated the release of GB to the extent of 0.7 times during the same exposure time. GA started to accumulate after 12 h exposure time.

These results thus indicate that KCl may help the release of BB, GA and GB into the cultured medium. Release of these metabolites from the cells may be due to the cellular lysis or damage of cell membrane as a result of the stress by KCl, as well as secretion from the cells. In particular, KCl promotes the release of BB, rather than GA and GB. In many plant, secondary metabolites produced by cell cultures have been reported to be accumulated intracellularly. It may be possible to produce much higher level of products if they are secreted into the medium. This is because the products that accumulate intracellularly sometimes may inhibit their own synthesis by regulation mechanisms such as product inhibition and repres-

sion. Releasing mechanism is however not yet clear. In this study, release of ginkgolides and bilobalide on KCl treatment may be caused by salt stress. Therefore, detailed research on the understanding about metabolite releasing mechanisms by ginkgo cell cultures is required.

Effect of KCl treatment on the total production of bilobalide and ginkgolides in cells and cultured medium

The KCl treatment influenced variation in total phytochemicals BB, GA and GB in cells and cultured medium (Figure 5). At KCl 50 mM treatment only GA production decreased compared to the control. The levels of BB and GB increased in cells and cultured medium at all KCl concentrations. After treatment with KCl 800 mM, the BB content in cells and cultured medium increased and reached a maximum level after 48 h of exposure time. Although KCl 800 mM inhibited cell growth, the maximum content of GA and GB in cells and cultured medium was obtained in the treatment of KCl 800 mM, which was 1.5 and 2.4 times higher than the control. In the present study, the effect of KCl on the production of BB, GA and GB in cells and cultured medium was maximal at treatment of KCl 800 mM and at 48 h of exposure time. However, effect of KCl on metabolite production at most of concentrations except for 800 mM were dependent on metabolite such as BB, GA and GB. In other words, the optimal concentration in BB, GA and GB production was different respectively, except for KCl 800 mM. Therefore the optimal concentration of salt required to elicit metabolite production varies depending on the metabolite type. Another important factor that determines the metabolite concentrations is the exposure time. Therefore, to achieve maximum stimulation of BB, GA and GB biosynthesis, both the concentrations and the exposure times of the elicitors need to be optimized.

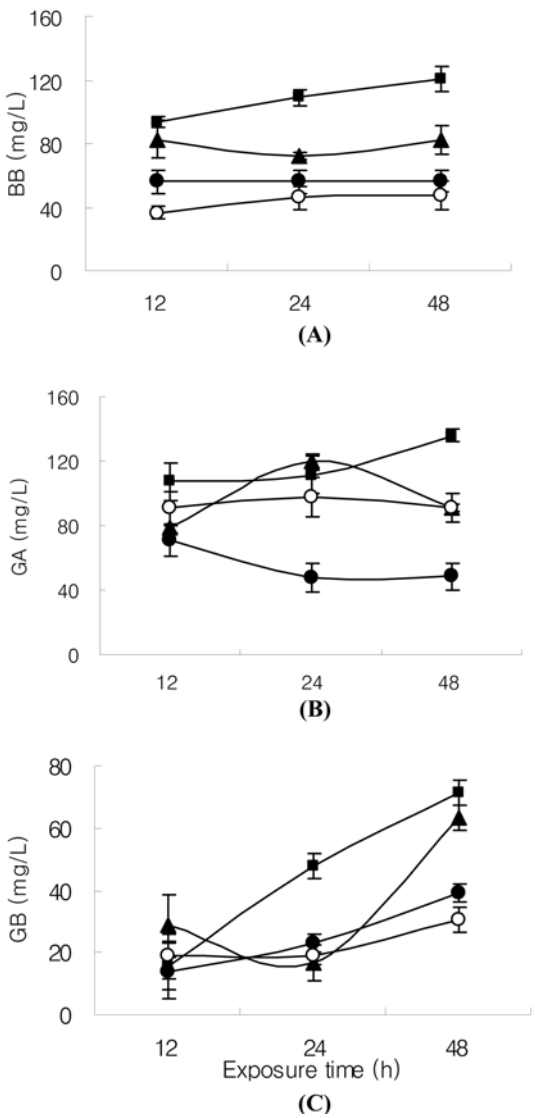

Figure 5. Total content of bilobalide (A), ginkgolide A (B) and ginkgolide B (C) in cells and cultured medium by various concentrations treatment of KCl. (●: 50 mM KCl, ▲: 200 mM KCl, ■: 800 mM KCl,○: control).

Several enzymes of secondary metabolism undergo regulation under the influence of several factors such as Mg, Ca, and Na (Lee, 2002). In our study since KCl enhanced the production of BB, GA and GB we think that the key enzymes in terpenoids metabolism undergo upregulation. Also, other researcher reported that KCl treatment was increase secondary metabolites. KCl is shown to induce accumulation of betains in *Lycium chinense* Mill (Kim and Rho., 1998), and ginsenosides from *Panax ginseng* (In *et al.*, 2006).

On the basis of our experiments with *G. biloba* cell cultures it is suggested that KCl salt elicitation can lead to high accumulation of BB, GA and GB in both cells and cultured medium. These results can go a long way in designing the strategy for large scale production of secondary metabolites from cell cultures. It would be an advantage if the metabolites are secreted into the medium as it would allow the industrial production by continuous culture system. However the investigation on the related defense and terpenoid biosynthesis mechanisms involved during the salt stress remain to be explored.

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